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Improved Prenatal Diagnosis of Congenital Human Cytomegalovirus Infection by a Modified Nested Polymerase Chain Reaction

Maria Grazia Revello,* Antonella Sarasini, Maurizio Zavattoni, Fausto Baldanti, and Giuseppe Gerna

Viral Diagnostic Service, IRCCS Policlinico San Matteo, Pavia, Italy

Two major variables may cause false-negative results in prenatal diagnosis of congenital human cytomegalovirus (HCMV) infection: sensitivity of the technique(s) used; and time elapsed between maternal infection and antenatal testing. Previous results indicated that rapid HCMV isolation from amniotic fluid samples and viral DNA detection in amniotic fluid by nested polymerase chain reaction (nPCR) had comparable levels of sensitivity (69.2% and 76.9%, respectively). The nPCR protocol was reviewed following two additional false-negative antenatal diagnosis in a twin pregnancy during which two procedures were performed at 18 and 23 weeks of gestation, respectively. In the new assay, multiple (instead of single) and 100 (instead of 20) µl amniotic fluid aliquots were individually amplified and tested by nPCR. By using this approach, low DNA levels (1–10 genome equivalents) were detected in 1–5/8 replicates of amniotic fluid samples taken from both twins during both procedures. In addition, viral DNA was detected in 5/6 replicates from two amniotic fluid samples still available from two previous false-negative cases. However, nPCR on multiple amniotic fluid replicates did not anticipate positive prenatal results in a retrospective case, which required two procedures for correct diagnosis and, when prospectively employed, did not avoid one additional false-negative prenatal diagnosis 8 weeks after maternal infection. Thus, delayed intrauterine transmission of the infection may be a potential cause of false-negative results. However, the combination of a very sensitive technique with appropriate timing of prenatal testing can substantially increase the reliability of prenatal diagnosis results. ***J. Med. Virol. 56:99–103, 1998.***

KEY WORDS: PCR; human cytomegalovirus (HCMV); amniotic fluid; DNA

INTRODUCTION

Incidence of congenital human cytomegalovirus (HCMV) infection varies from 0.3% in United Kingdom [Peckham et al., 1983] to 0.2–2.2% in the United States [Britt and Alford, 1996]. Only infants born to mothers with primary infection may be symptomatic at birth in up to 18% cases [Fowler et al., 1992]. Intrauterine transmission of HCMV infection occurs in about 50% of fetuses following primary infection in mothers [Britt and Alford, 1996]. Little is known about the mechanisms of transmission and the natural history of congenital HCMV infection. However, once infected, the fetus excretes HCMV via urine into the amniotic fluid. Culture of amniotic fluid has been shown to be the most reliable approach to prenatal diagnosis compared to determination of virus-specific IgM [Hohlfeld et al., 1991; Grose et al., 1992; Lamy et al., 1992; Donner et al., 1993; Nicolini et al., 1994; Mulongo et al., 1995; Revello et al., 1995]. However, concerns still remain about the predictive value of a negative amniotic fluid culture given a few reported cases of false-negative results [Catanzarite and Dankner, 1993; Donner et al., 1993; Nicolini et al., 1994; Mulongo et al., 1995; Revello et al., 1995].

It was demonstrated previously that the sensitivity of prenatal diagnosis of congenital HCMV infection was only slightly improved by using either single-step or nested polymerase chain reaction (nPCR) for detection of viral DNA in amniotic fluid [Revello et al., 1995]. In that study, neither virus isolation from nor viral DNA detection in amniotic fluid by the conventional nPCR described could avoid three false-negative prenatal diagnosis out of 13 intrauterine infections diagnosed at birth. Following an additional false-

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*Correspondence to: Maria Grazia Revello, Viral Diagnostic Service, IRCCS Policlinico San Matteo, 27100 Pavia, Italy.

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TABLE I. Virologic Findings and Results of Prenatal Diagnosis in Six Fetuses With Congenital HCMV Infection Diagnosed at Birth[a]

Fetus number	Weeks of gestation: HCMV infection in mother	Weeks of gestation: Prenatal diagnosis	HCMV isolation from amniotic fluid	HCMV antigenemia/ viremia on FPBL	Virus-specific IgM	nPCR on amniotic fluid: Conventional	nPCR on amniotic fluid: New	Outcome
1	18	21	negative	negative	negative	negative	positive[b]	CI
2	9	20	negative	ND	ND	negative	positive[b]	CI
3, left twin	11	18	negative	negative	negative	negative	positive[b]	
		23	negative	negative	negative	negative	positive[b]	CI
4, right twin	11	18	negative	negative	negative	negative	positive[b]	
		23	negative	negative	negative	negative	positive[b]	CI
5	4	18	negative	ND	ND	negative	negative	
		23	positive	positive	positive	positive	positive	CI
6	15	23	negative	negative	negative	ND	negative	CI

[a]FPBL, fetal peripheral blood leukocytes; nPCR, nested polymerase chain reaction; CI, congenital infection; ND, not done.
[b]Positive results were obtained retrospectively.

negative prenatal diagnosis in a set of twins, the conventional nPCR protocol was reviewed and modified. The new nPCR assay was evaluated on retrospective HCMV-negative amniotic fluid samples from fetuses subsequently found to be either infected (false-negative prenatal diagnosis) or uninfected (true-negative prenatal diagnosis) and on prospective amniotic fluid samples from 10 consecutive fetuses with known virologic outcome at birth.

MATERIALS AND METHODS

Polymerase Chain Reaction

PCR was carried out essentially according to a procedure described previously [Gerna et al., 1994; Revello et al., 1995]. Briefly, an HCMV genome sequence relevant to exon 4 of the major IE gene (IE1) was amplified. An internal control of amplification consisting of a fixed amount (100 genome equivalents, GE) of a recombinant DNA molecule (pAC2) flanked by the target sequence of outer primers (N1, nt 941–959; N2, 1431–1412) used for viral DNA amplification in clinical samples was routinely coamplified to detect PCR inhibitors. PCR products of IE1 region and pAC2 were detected by different molecular size. Samples negative for HCMV DNA but competent for amplification, as shown by PCR product of pAC2, were submitted to a second (nested) step of amplification using an inner set of primers (CM1, nt 1073–1092; CM2, nt 1336–1317). By using single-step PCR, samples containing >10 GE were consistently amplified, whereas samples containing 1–10 GE could be detected in the nested assay (nPCR). An arbitrary value of 5 GE was assigned to samples positive only by nPCR.

In the conventional nPCR procedure, a single aliquot of 50 μl amniotic fluid was used for DNA extraction by a rapid method based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidinium thiocyanate and the nucleic acid binding properties of silica particles [Boom et al., 1990]. A volume corresponding to 20 μl amniotic fluid was then amplified. On the other hand, in the modified nPCR test, DNA was extracted from 300–800 μl amniotic fluid (depending on the available amount of the sample) and multiple 100μl amniotic fluid replicates were then independently amplified. In both protocols, 1/100 of the first-round product volume was further amplified for 40 cycles using the thermal conditions previously described [Zipeto et al., 1993]. A single aliquot of 1×10^5 peripheral blood leukocytes (PBLs) was routinely examined to determine viral DNA in PBL (leukoDNAemia).

Patients and Samples

Two groups of amniotic fluid samples were tested by the new nPCR assay. The first group included 30 retrospective amniotic fluid samples (from 21 fetuses and 20 mothers) negative by virus isolation and conventional nPCR. Of these specimens, seven were from five fetuses (no. 1–5, Tables I and II) in whom congenital HCMV infection was detected only at birth. The remaining 23 retrospective amniotic fluid samples were taken from 16 fetuses in whom there was no evidence of antenatal viral infection, confirmed at birth by negative virus isolation from urine [Revello et al., 1995]. These samples were used to assess the specificity of the new nPCR test. Three (no. 1, 2, and 5, Tables I and II) of the five fetuses with false-negative prenatal diagnosis have been described previously [Revello et al., 1995], whereas a short clinical history of the remaining two fetuses (one set of twins, no. 3 and 4, Tables I and II) is as follows. A primary HCMV infection was diagnosed in a 33-year-old woman at 11 weeks' gestation with a twin monochorionic biamniotic pregnancy. A first prenatal procedure was undertaken at 18 weeks of gestation when amniotic fluid and fetal blood samples from both twins were found to be negative for virus isolation and viral DNA by conventional nPCR as well as for virus-specific IgM antibody. Five weeks later, a second procedure confirmed the negative results. After 36 weeks of gestation, two female twins were delivered. Physical examination revealed no abnormalities. However, HCMV was isolated at birth from the urine of both newborns.

The second group of samples included seven culture-

TABLE II. Detailed Results of the New nPCR Assay on Multiple Amniotic Fluid Replicates[a]

Fetus number	Prenatal diagnosis	HCMV maternal DNAemia (number of GE)	Number of HCMV-positive amniotic fluid replicates/number of examined by new nPCR	HCMV isolation at birth
1	21[b]	positive (5)	5/6	positive
2	20	negative (0)	5/6	positive
3, left twin	18	positive (5)	3/8	
	23	positive (5)	5/8	positive
4, right twin	18	positive (5)	2/8	
	23	positive (5)	1/6	positive
5	18	ND	0/7	
	23	ND	3/3[c]	positive
6	23	negative (0)	0/6	positive
7–13	19–30	positive (5–398)	0/3	negative
14–28	17–32	negative (0)	0/3	negative

[a]nPCR, nested polymerase chain reaction; GE, genome equivalents; ND, not done.
[b]Weeks of gestation.
[c]Positive by single-step PCR.

negative and three culture-positive amniotic fluid samples from 10 fetuses that were only tested prospectively by the new nPCR assay. Congenital infection was diagnosed at birth in four cases (one case of false-negative prenatal diagnosis, fetus no. 6, Tables I and II) and excluded in the remaining six. Thus, altogether, 40 amniotic fluid samples from 31 fetuses were examined.

Fetal blood samples were obtained from 25 fetuses (17 retrospective and 8 prospective cases) and PBL examined for viral DNA (leukoDNAemia) and/or HCMV pp65 antigenemia/viremia, depending on the number of PBLs available. HCMV-specific IgM were determined when prenatal diagnosis was carried out later than 18 weeks of gestation. All 30 pregnant women (20 included in the retrospective and 10 in the prospective study) who underwent prenatal diagnosis procedures had a primary HCMV infection diagnosed during pregnancy. Maternal diagnosis was documented by seroconversion in 13 cases, by clinical symptoms (fever, myalgia), and/or abnormal liver enzyme values and presence of virus-specific IgM in 13 cases, and by presence of IgM and pp65 antigenemia or leukoDNAemia [Revello et al., 1998] in the remaining four cases. Maternal PBLs were collected from 29 women at the time of amniocentesis and examined for presence of viral DNA. All newborns were examined for HCMV in urine within the first week of life.

Virus Isolation/Detection and Virus-Specific Antibody Determination

Early virus identification was achieved 18–24 hr after inoculation of clinical samples (amniotic fluid, urine, PBL) onto confluent human embryonic lung fibroblast monolayers grown in shell vials, using the indirect immunofluorescence technique and a monoclonal antibody to the major immediate early protein p72 [Gerna et al., 1990]. Fixed amounts (2×10^5 cells) of PBL were used for viremia determination [Gerna et al., 1990] and for cytospin preparations for direct detection of HCMV pp65 (antigenemia) [Gerna et al., 1992b]. HCMV-specific IgG [Gerna et al., 1992a] and IgM [Revello et al., 1991] were determined according to published procedures.

RESULTS

Using virus isolation from and/or conventional nPCR on amniotic fluid, false-negative prenatal results were obtained in five fetuses (Table I, no. 1–4, 6), including one set of twins sampled twice at 18 and 23 weeks of gestation (fetus 3 and 4). Four fetuses (no. 1, 3, 4, 6) were also negative for HCMV viremia/antigenemia as well as for presence of virus-specific IgM. When amniotic fluid samples were tested retrospectively by the new nPCR procedure, viral DNA was detected in 4/5 amniotic fluid samples, providing a correct prenatal diagnosis of congenital HCMV infection in four fetuses (Table I, no. 1–4). However, the new nPCR assay did not avoid the need for a second sampling prior to achieve a correct prenatal diagnosis (Table I, no. 5). In this case, first amniocentesis was performed at 18 weeks' gestation (14 weeks after maternal infection) and HCMV was not detected by any technique (including new nPCR on amniotic fluid), whereas 5 weeks later (23 weeks' gestation), HCMV was readily isolated from amniotic fluid and viral DNA detected by single-step PCR. Moreover, the fetus was viremic and virus-specific IgMs were detected in fetal serum. In addition, viral DNA could not be detected in the amniotic fluid sample of the remaining fetus (no. 6), examined prospectively by new nPCR 8 weeks after maternal infection.

Detailed results obtained by the new nPCR procedure on multiple amniotic fluid replicates are reported in Table II. Of the six amniotic fluid replicates that were tested for fetuses no. 1 and 2, five were positive for viral DNA by nPCR (5 GE). Similarly, in the twin pregnancy (fetuses no. 3 and 4), 3/8 and 5/8 replicates

from the left twin during the first and second procedure, respectively, and 2/8 and 1/6 replicates of sequential amniotic fluid samples from the right twin were found to contain low amounts of viral DNA, which remained stable in both twins during the 5 weeks elapsed between the two procedures. On the other hand, all seven replicates of the first amniotic fluid sample obtained from fetus no. 5 were negative for viral DNA, whereas 3/3 replicates of the amniotic fluid sample obtained during the second procedure were DNA-positive by single-step PCR. Finally, 0/6 amniotic fluid replicates were positive in the fetus that was prospectively examined only by the new nPCR test (no. 6) and found congenitally infected at birth.

In order to investigate whether low levels of viral DNA detected by the new nPCR assay could represent contamination caused by the invasive procedure, maternal PBLs obtained at the time of amniocentesis were examined for viral DNA. As reported in Table II, nine women (including the mother of twins no. 3 and 4) had a positive DNAemia at the time of amniocentesis, although at a very low level (median value 5 GE, range 5–398). Of these, only two were mothers of fetuses with amniotic fluid positive by new nPCR (fetuses no. 1, 3, and 4), whereas the remaining seven, including a woman with the highest level of leukoDNAemia (398 GE), were mothers of fetuses with amniotic fluid samples negative by new nPCR (fetuses no. 7–13). From these results, it seems unlikely that concomitant maternal leukoDNAemia was the source of viral DNA detected in amniotic fluid through the invasive procedure.

The specificity of the new nPCR assay was 100%, as shown by negative results on 3×100 μl replicates of 29 amniotic fluid samples (23 retrospective and 6 prospective) from 22 pregnant women with primary HCMV infection who did not transmit the infection to their fetuses (no. 7–28, Table II).

Finally, viral DNA was detected by single-step PCR in 3/3 amniotic fluid replicates from three fetuses prospectively tested by the new nPCR test (data not shown). Congenital HCMV infection was confirmed at birth (2 cases) or after termination of pregnancy (1 case).

DISCUSSION

A reliable prenatal diagnosis of congenital HCMV infection remains a primary goal and challenge in a diagnostic virology laboratory. In particular, increasing the sensitivity is important given the few reported false-negative results [Catanzarite and Dankner, 1993; Donner et al., 1993; Nicolini et al., 1994; Mulongo et al., 1995; Revello et al., 1995]. It was shown recently that the sensitivity of prenatal diagnosis for HCMV infection was slightly improved by the use of either single-step or nested PCR for viral DNA detection in amniotic fluid [Revello et al., 1995]. In that study, 10/13 (76.9%) congenital infections were diagnosed by PCR and 9/13 (69.2%) by virus isolation. However, the occurrence of a false-negative prenatal diagnosis involving a twin pregnancy sampled twice at 18 and 23 weeks of gestation led us to modify the conventional nPCR assay previously described and routinely employed. In particular, in the new nPCR, the volume of amniotic fluid tested was increased by 15–40 times with respect to the former conventional nPCR protocol (300–800 μl vs. 20 μl). Retrospective testing of amniotic fluid samples still available from four fetuses, including the set of twins with false-negative prenatal diagnosis results, showed that low levels (5 GE) of HCMV DNA could indeed be detected in a variable number of amniotic fluid replicates of all fetuses. The low quantity of DNA detected by the new nPCR protocol provides a plausible reason for the negative results obtained when a single 20 μl aliquot of amniotic fluid was amplified. Indeed, increasing the volume of amniotic fluid had been shown to increase the sensitivity of a reverse transcription PCR assay for prenatal diagnosis of rubella virus infection [Revello et al., 1997]. Similarly, increasing the amount of cerebrospinal fluid tested was reported to improve sensitivity of PCR for diagnosis of herpes simplex encephalitis [Aurelius et al., 1991].

The new nPCR, however, failed to detect viral DNA in one fetus (no. 6) sampled at 23 weeks of gestation (8 weeks after maternal infection) and did not anticipate the diagnosis in one additional fetus (no. 5) first sampled at 18 weeks of gestation (14 weeks after maternal infection). In the latter case, fetal HCMV infection was readily diagnosed 5 weeks later by virus isolation from both amniotic fluid and PBL as well as by presence of virus-specific IgM. These findings seem to indicate that time interval between maternal infection and fetal sampling remains an important factor, affecting the reliability of prenatal diagnosis [Mulongo et al., 1995; Revello et al., 1995]. Therefore, procedures should be delayed as long as possible, with respect to maternal infection, to reduce the risk of false-negative results due to delayed intrauterine transmission, and, when indicated, a second procedure should be considered.

In this study, the number of amniotic fluid replicates tested to assess the sensitivity was higher ($n = 6$–8) than that used to determine the specificity of the new nPCR assay ($n = 3$). This was dictated by the amount of amniotic fluid available. However, in order to avoid the risk of false-negative results when only three replicates are tested (as in fetus no. 4 of Table II), at least six replicates of amniotic fluid samples are currently amplified in the routine prenatal diagnosis protocol.

The detection of low amounts of viral DNA lingering in amniotic fluid in the absence of virus isolation and fetal infection is an intriguing finding. It has been suggested that a possible route of intrauterine transmission may involve the infection of placental tissue first and then amniotic cells that would subsequently be ingested by the fetus [Grose and Weiner, 1990]. However, in our experience, primary cultures of amniotic cells do not seem to be susceptible to HCMV infection when inoculated with amniotic fluid and urine samples from HCMV congenitally infected fetuses/newborns

(data not shown). Thus, the origin of the very low DNA levels observed to persist for a few weeks in the amniotic fluid of apparently uninfected fetuses remains as yet unexplained.

Finally, it is important to stress that concomitant presence of viral DNA in maternal PBL does not seem to represent a source of the viral DNA detected in amniotic fluid through invasive procedures. This finding also implies that iatrogenic transmission of the infection seems unlikely, as recently described [Revello et al., 1998].

In conclusion, nPCR on multiple amniotic fluid replicates appears to be the most sensitive and specific virologic test for antenatal diagnosis of congenital HCMV infection. However, the apparently erratic behavior of HCMV and the poor knowledge of the natural history of intrauterine HCMV infection continue to hamper a reliable prenatal diagnosis. In addition, the factors predictive of poor outcome following maternal infection remain to be identified.

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